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Research Article

The Role of Neurotransmitters in Protection against Amyloid-β Toxicity by KiSS-1 Overexpression in SH-SY5Y Neurons

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Recent studies have suggested that the kisspeptin (KP) and kissorphin (KSO) peptides have neuroprotective actions against the Alzheimer's amyloid-β (Aβ) peptide. Overexpression of the human KiSS-1 gene that codes for KP and KSO peptides in SH-SY5Y neurons has also been shown to inhibit Aβ neurotoxicity. The *in vivo* actions of KP include activation of neuroendocrine and neurotransmitter systems. The present study used antagonists of KP, neuropeptide FF (NPFF), opioids, oxytocin, estrogen, adrenergic, cholinergic, dopaminergic, serotonergic, and γ-aminobutyric acid (GABA) receptors plus inhibitors of catalase, cyclooxygenase, nitric oxide synthase, and the mitogen activated protein kinase cascade to characterize the KiSS-1 gene overexpression neuroprotection against Aβ cell model. The results showed that KiSS-1 overexpression is neuroprotective against Aβ and the action appears to involve the KP or KSO peptide products of KiSS-1 processing. The mechanism of neuroprotection does not involve the activation of the KP or NPFF receptors. Opioids play a role in the toxicity of Aβ in the KiSS-1 overexpression system and opioid antagonists naloxone or naltrexone inhibited Aβ toxicity. The mechanism of KiSS-1 overexpression induced protection against Aβ appears to have an oxytocin plus a cyclooxygenase dependent component, with the oxytocin antagonist atosiban and the cyclooxygenase inhibitor SC-560 both enhancing the toxicity of Aβ.

1. Introduction

Recent studies have suggested that the kisspeptin (KP) and kissorphin (KSO) peptide derivatives of the metastasis-suppressor KiSS-1 gene may have neuroprotective actions against the Alzheimer's amyloid-β (Aβ) peptide [1]. The studies have also suggested that stable overexpression of the KiSS-1 gene in SH-SY5Y neurons creates a cell line that is resistant to the neurotoxicity of Aβ [1]. The primary role of KP peptides is as a regulator of hypothalamic-pituitary-gonadal- (HPG-) axis via stimulation of gonadotrophin-releasing hormone (GnRH) release [2]. The KP peptides are ligands for the GPR-54 receptor [3–7] and the neuropeptide FF (NPFF) receptors, NPFFR1 (GPR-147) and NPFFR2 (GPR-74) [3, 4, 6–9]. The KSO peptides have been suggested to be ligands for the NPFF receptors but not the GPR-54 receptor [10]. Both KP and KSO peptides are protective against the Aβ peptide *in vitro* [1]. However, the neuroprotective actions of KP and KSO peptides have been suggested not to be mediated via actions on GPR-54 or NPFF receptors [1]. Fibrillar Aβ peptides stimulate the release of KP peptides [1, 11] and KP has been suggested to colocalize with Aβ deposits in the Alzheimer's brain [11].

The actions of KP peptides are thought to be mediated via activation of either GPR-54 or NPFF receptors. However, *in vivo* actions on the opioid system [12, 13], oxytocin/vasopressin systems [4, 14, 15], neurotransmitter systems [16, 17], activation of endogenous antioxidants [18], activation of nitric oxide [17], and possible activation of prostaglandin synthesis [19] have not been tested with GPR-54 or NPFF receptor antagonists.

The present study was conducted to characterize a model of KiSS-1 gene overexpression neuroprotection against Aβ in SH-SY5Y neurons *in vitro* [1] and to determine the role of neurotransmitter systems in the neuroprotection. The effects

of antagonists of KP, NPFF, opioids, oxytocin, estrogen, adrenergic, cholinergic, dopaminergic, serotonergic, and γ-aminobutyric acid (GABA) receptors were tested. Inhibitors of catalase, cyclooxygenase, nitric oxide synthase, and the mitogen activated protein kinase cascade were also tested.

2. Materials and Methods

2.1. Materials. Synthetic Aβ peptides plus anti-kisspeptin antibody were obtained from Bachem. Human SH-SY5Y neuroblastoma cell line was obtained from the Health Protection Agency Cell Culture Collection. ASCAT peptide was obtained from Insight Biotechnology Ltd. 3-Amino-1,2,4-triazole, atosiban, atropine sulphate, 1(S),9(R)-(−)-bicuculline methiodide, BTA-EG4 hydrate, cyproheptadine hydrochloride, DAPT, haloperidol, KP234, mecamylamine hydrochloride, methysergide maleate, naltrexone, N^G-Methyl-L-arginine acetate salt, PD98059, phenoxybenzamine hydrochloride, prazosin hydrochloride, propranolol hydrochloride, RF9, SC-560, tamoxifen, and yohimbine hydrochloride, plus all other chemicals, were obtained from Sigma-Aldrich.

2.2. Aβ Fibril Formation. Batches of synthetic Aβ 1–40 or Aβ 25–35 were dissolved in distilled water at a concentration of 1.0 mg/mL and incubated at 37°C for 24 h, with constant oscillation. Following incubation, the formation of fibrils was confirmed by TEM or Congo red assay as previously described by Milton and Harris [20–22].

2.3. Cell Cultures and KiSS-1 Overexpression. Human SH-SY5Y neuroblastoma cells were routinely grown in a 5% CO_2 humidified incubator at 37°C in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and HAM's F12 with Glutamax (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, penicillin (100 units/mL), and streptomycin (100 mg/mL) [23]. The human KiSS-1 cDNA clone (NM_002256) was obtained from Origene and PCR cloned into the pcDNA4/TO/myc-His expression vector using forward (5′-TTAGGATCCATGAACTCACTGGTTTCTTGGCA-3′) and reverse (5′-ATACTCGAGGCCCCGCCCAGCGCTTCT-3′) oligonucleotides to create the PKiSS expression vector. SH-SY5Y cells were transfected with PKiSS or control vector using lipofectamine (Invitrogen), and stably expressing clones were selected by culturing in 100 μg/mL Zeocin (Invitrogen). The presence of KiSS-1 overexpression was confirmed by immunocytochemistry and RT-PCR analysis. Human neuroblastoma SH-SY5Y, PKiSS, and PVect cells were cultured in 96-well plates and differentiated with retinoic acid for 7 days prior to experimentation.

2.4. Immunocytochemistry. Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized in ice cold methanol for 30 min. Cells were incubated in block solution (10% bovine serum albumin in PBS) for 15 min, followed by incubation with primary antibody anti-KP 45–54 (1 : 1000) in block solution for 1 h. Primary antibody was removed followed by 3 × 5 min washes in PBS, prior to incubation with goat anti-rabbit IgG-Alexa-fluor 488 secondary (Abcam PLC, Cambridge; 1 : 500) in block solution for 45 min. Secondary antibody was removed and cells were washed 3 times in PBS. Cells were incubated with 100 μg/mL RNase A for 20 min at 37°C, followed by 3 × 5 min washes and incubation with 1 μM TO-PRO-3 Iodide (642/661; Invitrogen) for 20 min. Cells were washed 3 times in PBS and fluorescence was visualized by sequential scanning using a Leica TCS SP2 confocal system (Leica Microsystems, Milton Keynes, UK) [11].

2.5. Western Blotting of Conditioned Media. To determine the presence of KP released into the media from KiSS-1-overexpressing and vector control cells proteins were purified from 6 mls of conditioned media using an Amicon system (Merck Millipore UK). Proteins in extracts were resuspended in sample buffer before boiling for 5 min and separation of samples using a 15% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose membrane and membranes were blocked with 3% nonfat dried milk powder in PBS containing 0.1% Tween 20 (1 h at room temperature). Membranes were incubated overnight at 4°C with rabbit anti-KP 45–54 antibody. Unbound antibody was rinsed from the membranes before incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Immunoreactivity was detected using an enhanced chemiluminescence substrate and UVP BioImaging system.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR). To determine the steady-state levels of KiSS-1 mRNA, total RNA was isolated from KiSS-1-overexpressing and vector control cells using a Qiagen RNeasy extraction kit (Cat No: 74104) according to the manufacturer's instructions. RT-PCR was performed using the Qiagen one-step RT-PCR reagent kit (Cat. no: 210210) with KiSS-1 forward 5′-TTAGGATCCATGAACTCACTGGTTTCTTGGCA-3′ and reverse (5′-ATACTCGAGGCCCCGCCCAGCGCTTCT-3′) primers. The level of β-actin was used to normalize loadings of total RNA [4].

2.7. Effects of Neurotransmitter Antagonists. Test drugs were used at the following concentrations: anti-KP (10 μg/mL); KP234 (10 μM); RF9 (10 μM); ASCAT (100 μM); BTA-EG4 hydrate (10 μM); naloxone (1 μM); naltrexone (1 μM); atosiban (1 μM); phenoxybenzamine hydrochloride (10 μM); prazosin hydrochloride (250 nM); yohimbine hydrochloride (50 nM); propranolol hydrochloride (50 nM); atropine sulphate (10 μM); mecamylamine hydrochloride (10 μM); haloperidol (10 μM); cyproheptadine hydrochloride (10 nM); methysergide maleate (1 μM); 1(S),9(R)-(−)-bicuculline methiodide (50 μM); tamoxifen (10 μM); 3-Amino-1,2,4-triazole (50 mM); SC-560 (1 μM); N^G-Methyl-L-arginine acetate salt (1 mM) and PD98059 (50 μM). Stock solutions of at least 100x maximum required concentration for testing were prepared in PBS (anti-KP), ddH_2O (KP234, RF9, ASCAT, naloxone, naltrexone, atosiban, yohimbine hydrochloride, 1(S),9(R)-(−)-bicuculline methiodide, 3-Amino-1,2,4-triazole, N^G-Methyl-L-arginine acetate salt), methanol

(phenoxybenzamine hydrochloride, prazosin hydrochloride), ethanol (atropine sulphate, mecamylamine hydrochloride, cyproheptadine hydrochloride), or DMSO (BTA-EG4 hydrate, propranolol hydrochloride, haloperidol, methysergide maleate, tamoxifen, SC-560, PD98059) prior to dilution to the required concentration in cell culture media. On the day of the experiment 5×10^3 differentiated PKiSS expressing SH-SY5Y cells/well in 96-well plates were pretreated with either media alone (control) or test drugs for a 2 h period. The fibrillar Aβ 1–40 (10 μM) was then added to induce toxicity and cells were incubated for a further 16 hours prior to determination of cell viability. None of the solvents used (PBS, ddH2O, methanol, ethanol, or DMSO) had a statistically significant effect on cell viability or Aβ 1–40 (10 μM) toxicity at a 1 : 100 dilution in cell culture medium.

2.8. Cell Viability. After treatment with test peptides or drugs and incubation for the appropriate time the viability was determined by either trypan blue dye exclusion with at least 100 cells counted per well or by 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (MTT) reduction [24]. For MTT reduction determination, after incubation with test substances MTT (10 μL: 12 mM stock) was added and cells incubated for a further 4 hours. Cell lysis buffer [100 μL/well; 20% (v/v) SDS, 50% (v/v) N,N-dimethylformamide, pH 4.7] was added and after repeated pipetting to lyse cells the MTT formazan product formation was determined by measurement of absorbance change at 570 nm. Control levels in the absence of test substances were taken as 100% and the absorbance in the presence of cells lysed with Triton X-100 at the start of the incubation period with test substances taken as 0% [25]. All of the drugs tested had no statistically significant effect on the MTT assay in the absence or presence of cells. None of the solvents used (PBS, ddH2O, methanol, ethanol, or DMSO) had a statistically significant effect on the MTT assay in the absence or presence of cells.

2.9. Data Analysis. All data are expressed as means ± s.e.m. For cytotoxicity experiments data are expressed as % viable cells (trypan blue dye exclusion) or % control cells (MTT reduction). Statistical analysis was performed by one-way analysis of variance (ANOVA) due to the multiple variables (Aβ, test drug, and Aβ plus test drug being compared) using GraphPad Prism software (version 6). *Post hoc* analysis was carried with Tukey (for analysis of differences between KiSS-1 overexpressing and vector cells response to Aβ) or Dunnett (for comparisons involving test drugs) multiple comparison based on the recommendations of GraphPad Prism software for the data sets concerned, with a P value of <0.05 considered statistically significant.

3. Results

3.1. KiSS-1 Overexpression Cell Line Characterization. The overexpression of the human KiSS-1 gene in the PKiSS SH-SY5Y neurons, stably transfected with the pcDNA4/TO/myc-His expression vector containing the human KiSS-1 gene, was confirmed using immunocytochemistry (Figure 1(a)), which showed that the anti-KP 45–54 staining was found within the cytoplasm. The staining of PVect control cells, stably transfected with the pcDNA4/TO/myc-His expression vector, showed no anti-KP 45–54 staining above the background levels (Figure 1(b)). Conditioned media from PKiSS SH-SY5Y neurons and PVect control cells were collected and the presence of immunoreactive (ir) KP was determined by western blotting. Results showed the presence of an ir-KP low molecular weight band (<10 kDa) in media from PKiSS SH-SY5Y neurons, that was not found in PVect control cells (Figure 1(c)). To confirm that the transfected KiSS-1 gene was expressed cells were analyzed by RT-PCR. Results showed a high level of KiSS-1 mRNA in the PKiSS SH-SY5Y neurons compared to that found in naive (untransfected) SH-SY5Y neurons and PVect SH-SY5Y neurons (Figure 1(d)).

3.2. Neuroprotection against Amyloid-β by KiSS-1 Overexpression and the Role of Kisspeptin. The overexpression of the KiSS-1 gene in SH-SY5Y neurons was shown to be significantly ($P < 0.0001$; one-way ANOVA, Tukey post hoc test) neuroprotective against Aβ 25–35 toxicity (Figure 2(a)), in agreement with the previous studies [1]. Pretreatment with anti-KP (10 μg/mL), KP234 (10 μM), and RF9 (10 μM) was tested to confirm the observations from a previous study [1]. The anti-KP antibody significantly ($P = 0.0421$; one-way ANOVA, Dunnett post hoc test) enhanced the toxicity of Aβ 1–40 (10 μM) in KiSS-1 overexpressing neurons (Figure 2(b)), whilst neither the KP receptor antagonist KP234 [26] (Figure 2(c)) nor the NPFF receptor antagonist RF9 [27] (Figure 2(d)) had any significant effect, in agreement with previous studies [1]. The anti-KP antibody, KP234, and RF9 had no effect on the KiSS-1 overexpressing neurons alone. The doses of anti-KP antibody, KP234, and RF9 were selected based on previous studies [1]. These results suggest that KP is the neuroprotective component derived from the KiSS-1 gene, confirming previous studies [1], and indicate that the neuroprotective actions of KP are not mediated via actions on either the KP or NPFF receptors. The results further suggest that another mechanism, possibly via a different receptor or protein interactions between KP and Aβ, may play a role.

3.3. The Role of Amyloid-Binding Interactions in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptides that are suggested to be the neuroprotective derivatives from KiSS-1 overexpression have been shown to specifically bind Aβ. A synthetic peptide, ASCAT, which contains an Aβ-like sequence [24] and competes with Aβ binding to KP was therefore tested. The dose chosen (100 μM) has previously been shown to prevent Aβ inhibition of catalase without having a neuroprotective effect [24] and to prevent KP binding to amyloid peptides. Results showed that this compound had no significant effect on KiSS-1 overexpression induced neuroprotection against Aβ (Figure 3(a)). The BTA-EG4 compound, that has been developed as an Aβ binding agent [28], has previously been shown to displace Aβ binding to catalase [29] by binding the CAβBD of Aβ [24]. The dose chosen (10 μM) prevents Aβ interactions with catalase but is not neuroprotective itself [29]. The KP peptide has been

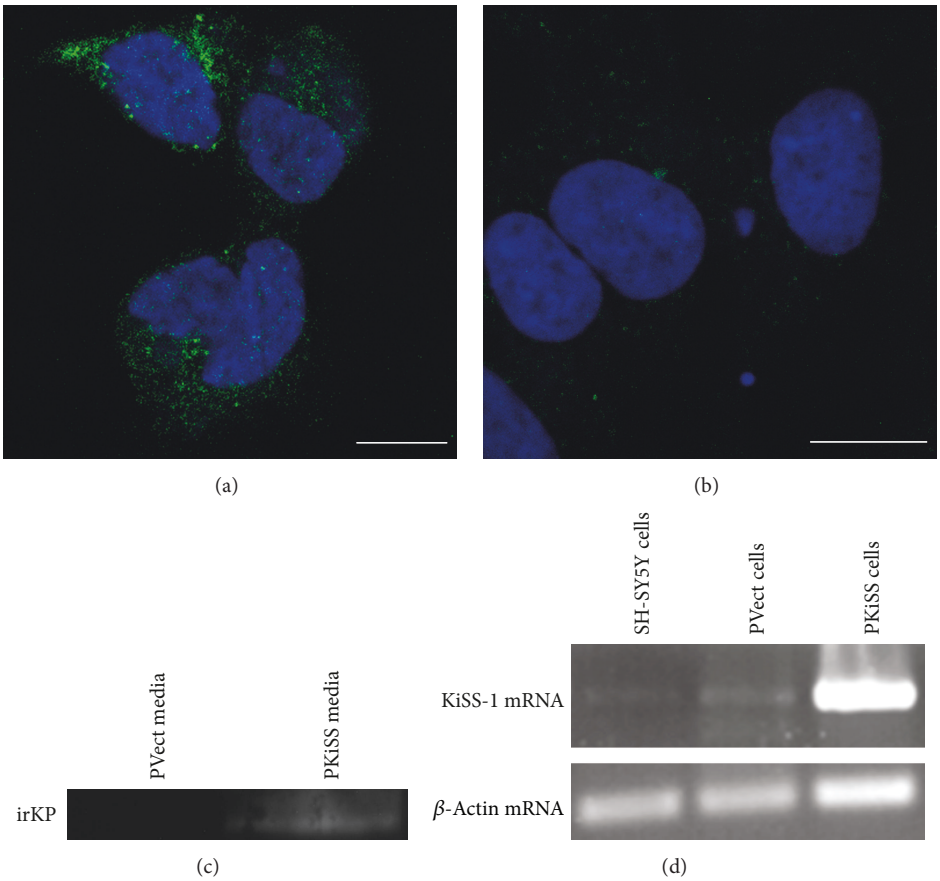

Figure 1: Characterization of KiSS-1 gene overexpression in SH-SY5Y neurons. (a) Immunocytochemistry of human SH-SY5Y neuron stable cell line containing the KiSS-1 gene vector (PKiSS) showing localization of kisspeptin in the cytoplasm. (b) Immunocytochemistry of human SH-SY5Y neuron stable cell line containing the pcDNA4/TO/myc-His expression vector (PVect) showing no localization of kisspeptin above background. KP appears green (anti-KP 45–54 staining) and the nucleus appears blue (TO-PRO-3 Iodide staining). Bars = 10 μm. (c) Western blot of conditioned media from PKiSS and PVect cells showing ir-KP staining of a <10 kDa band in PKiSS media. (d) RT-PCR analysis of KiSS-1 and β-actin mRNA in human SH-SY5Y neurons, human SH-SY5Y neuron stable cell line containing the pcDNA4/TO/myc-His expression vector (PVect), and human SH-SY5Y neuron stable cell line containing the KiSS-1 gene vector (PKiSS).

shown to bind the CAβBD of Aβ [1] and thus BTA-EG4 may displace KP binding to Aβ. When BTA-EG4 was tested in KiSS-1 overexpressing cells the compound had no effect of Aβ toxicity (Figure 3(b)). These results suggest that the mechanism for KiSS-1 neuroprotection against Aβ may not involve direct protein interactions between KP and Aβ.

3.4. The Role of Opioid Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. Opioids are neuroprotective against Aβ [30, 31] and also involved in KP activation of GnRH [12, 13]. The effects of the opioid receptor antagonists naloxone and naltrexone on KiSS-1 overexpression neuroprotection against Aβ were therefore tested. The doses of naloxone (1 μM) and naltrexone (1 μM) have previously been demonstrated to be effective in blocking the actions of opioids in cell culture models [30, 31]. Results showed that naloxone significantly ($P = 0.0230$; one-way ANOVA, Dunnett post hoc test) enhanced KiSS-1 overexpression neuroprotection against Aβ (Figure 4(a)). The naltrexone significantly ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) enhanced MTT reduction in control cells, suggesting that the compound had a proliferative effect on the KiSS-1 overexpressing neurons (Figure 4(b)). Naltrexone also significantly ($P = 0.0086$; one-way ANOVA, Dunnett post hoc test) enhanced KiSS-1 overexpression neuroprotection against Aβ (Figure 4(b)).

3.5. The Role of Oxytocin in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide is known to activate oxytocin *in vivo* [4]. The SH-SY5Y neurons express oxytocin receptors [32] and oxytocin has neuroprotective actions in this cell line [33]. The effects of atosiban, an antagonist of oxytocin [34], on KiSS-1 overexpression neuroprotection against Aβ were therefore tested at a dose (1 μM) that is known to be effective in cell culture models. Results showed atosiban significantly ($P = 0.0059$; one-way ANOVA, Dunnett post hoc test) enhanced the toxicity of Aβ in KiSS-1 overexpressing neurons (Figure 5). This suggests that the oxytocin receptor system may play a role in KiSS-1 mediated neuroprotection.

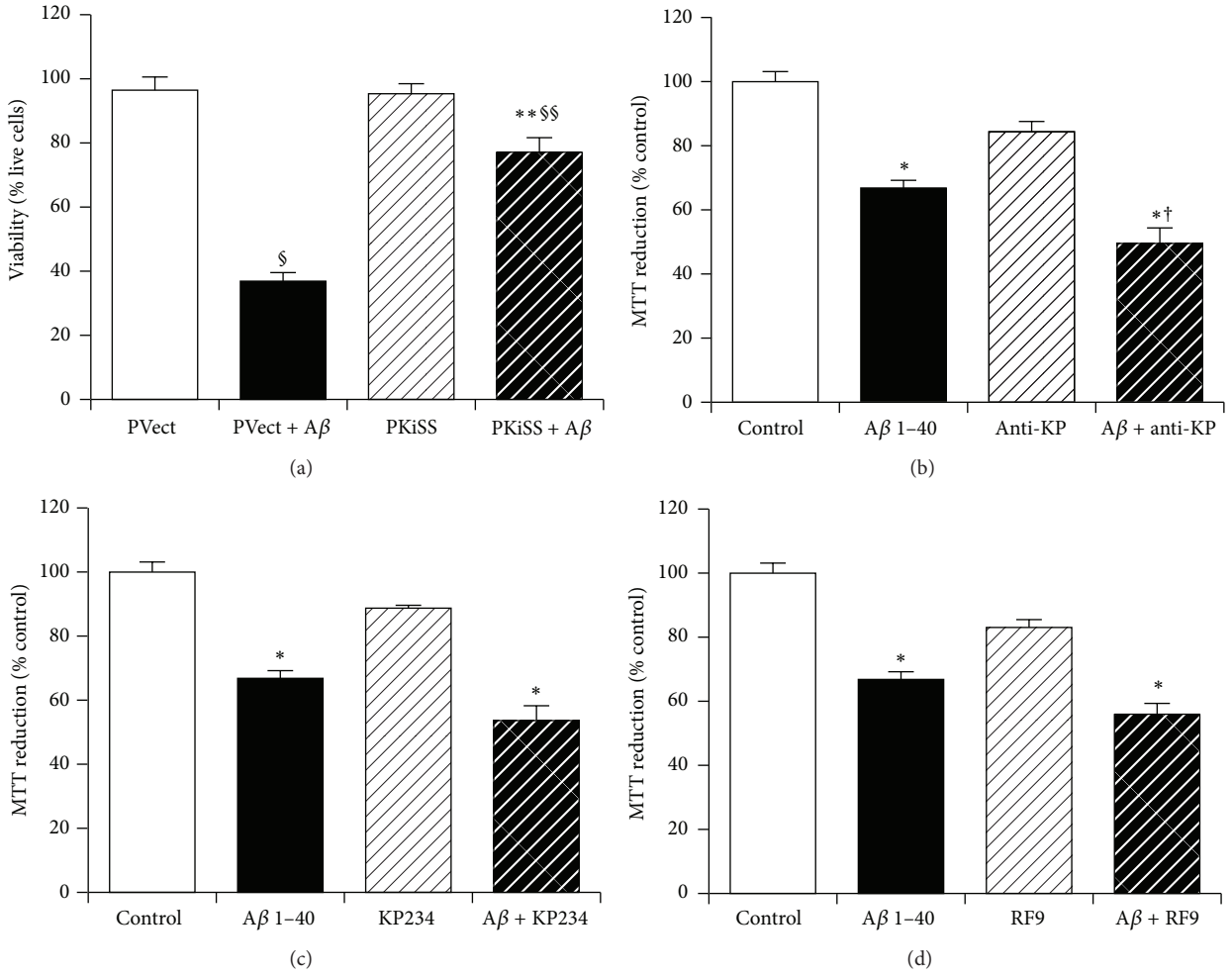

Figure 2: Effect of KiSS-1 gene overexpression on amyloid-β toxicity. (a) Human SH-SY5Y neurons stable cell lines containing control vector (PVect) or the KiSS-1 gene vector (PKiSS) were exposed to fibrillar Aβ 25–35 (10 μM) and cell viability determined by trypan blue exclusion. PKiSS cells were pretreated with (b) anti-kisspeptin antibody (Anti-KP: 10 μg/mL) or (c) kisspeptin receptor antagonist (KP234: 10 μM) or (d) neuropeptide FF receptor antagonist (RF9: 10 μM) for 2 h prior to exposure to fibrillar Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. (a) $^{\S}P < 0.05$ versus PVect; $^{**}P < 0.05$ versus PKiSS; $^{\S\S}P < 0.05$ versus PVect + Aβ; (b–d) $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

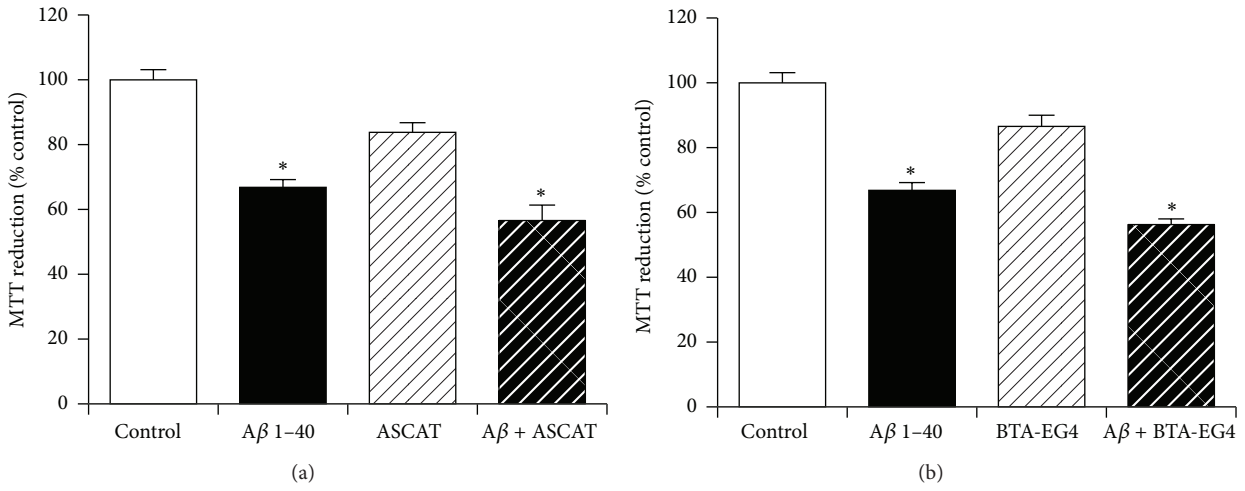

Figure 3: Effect of amyloid-binding compounds on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) ASCAT peptide (100 μM) or (b) BTA-EG4 (10 μM) for 2 h prior to exposure to fibrillar Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); one-way ANOVA.

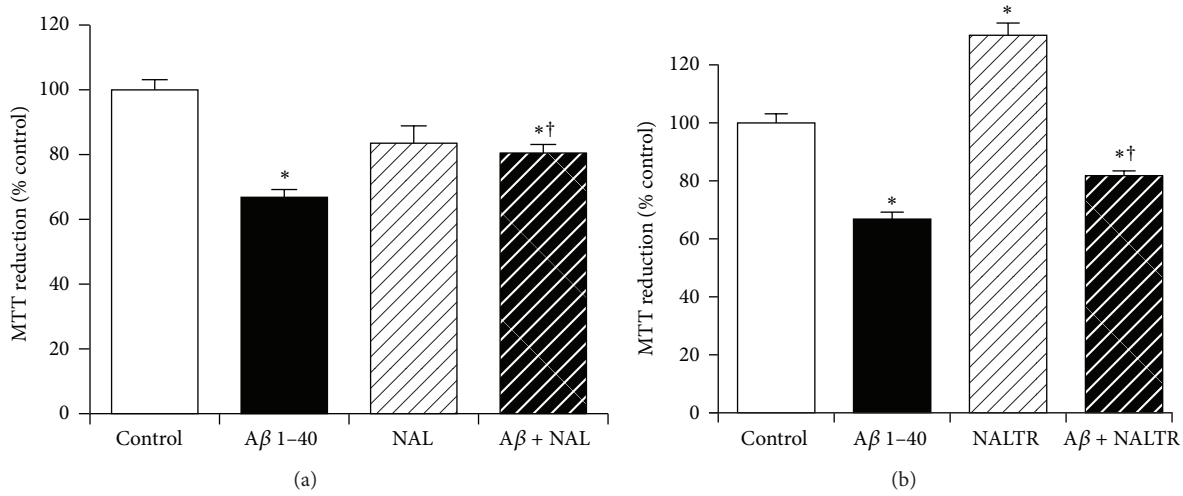

Figure 4: Effect of opioid receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) opioid antagonist naloxone (NAL: 1 μM) or (b) opioid antagonist naltrexone (NALTR: 1 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

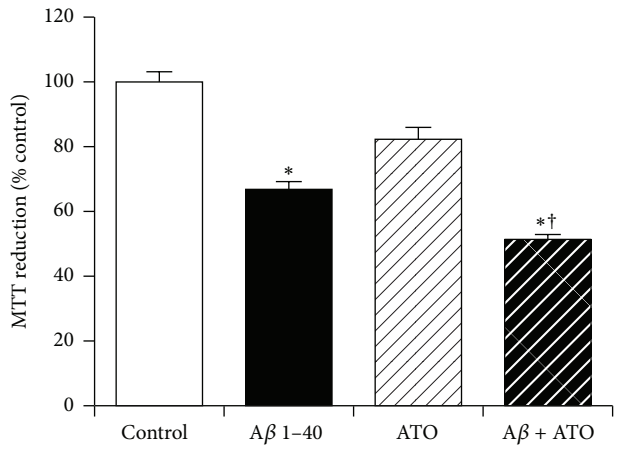

Figure 5: Effect of oxytocin receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with atosiban (ATO: 1 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

3.6. The Role of Adrenergic Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide facilitates passive avoidance learning and memory consolidation *in vivo*, which can be inhibited by both α- and β-adrenergic antagonists [17]. The KP peptide also has antidepressant-like activity that can be inhibited by α2-adrenergic antagonists [16]. The effects of α- and β-adrenergic antagonists on KiSS-1 overexpression neuroprotection against Aβ were therefore tested. The doses of phenoxybenzamine hydrochloride (10 μM), prazosin hydrochloride (250 nM), yohimbine hydrochloride (50 nM), and propranolol hydrochloride (50 nM) have previously been demonstrated to be effective in neuronal cell culture models. Results showed that the α-adrenergic antagonists phenoxybenzamine hydrochloride (Figure 6(a)), prazosin hydrochloride (Figure 6(b)), and yohimbine hydrochloride (Figure 6(c)) had no significant effect on the toxicity of Aβ in KiSS-1 overexpressing neurons. The β-adrenergic antagonist propranolol hydrochloride caused a significant ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) reduction in the viability of KiSS-1 overexpressing SH-SY5Y neurons (Figure 6(d)), at a dose that is nontoxic to SH-SY5Y neurons [35]. The propranolol also caused a significant ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) enhancement of Aβ toxicity in the KiSS-1 overexpressing neurons (Figure 6(d)), suggesting that the toxicity of propranolol in the KiSS-1 overexpressing cells was additive to the toxicity of Aβ.

3.7. The Role of Cholinergic Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide facilitates passive avoidance learning and memory consolidation *in vivo*, which can be inhibited by muscarinic

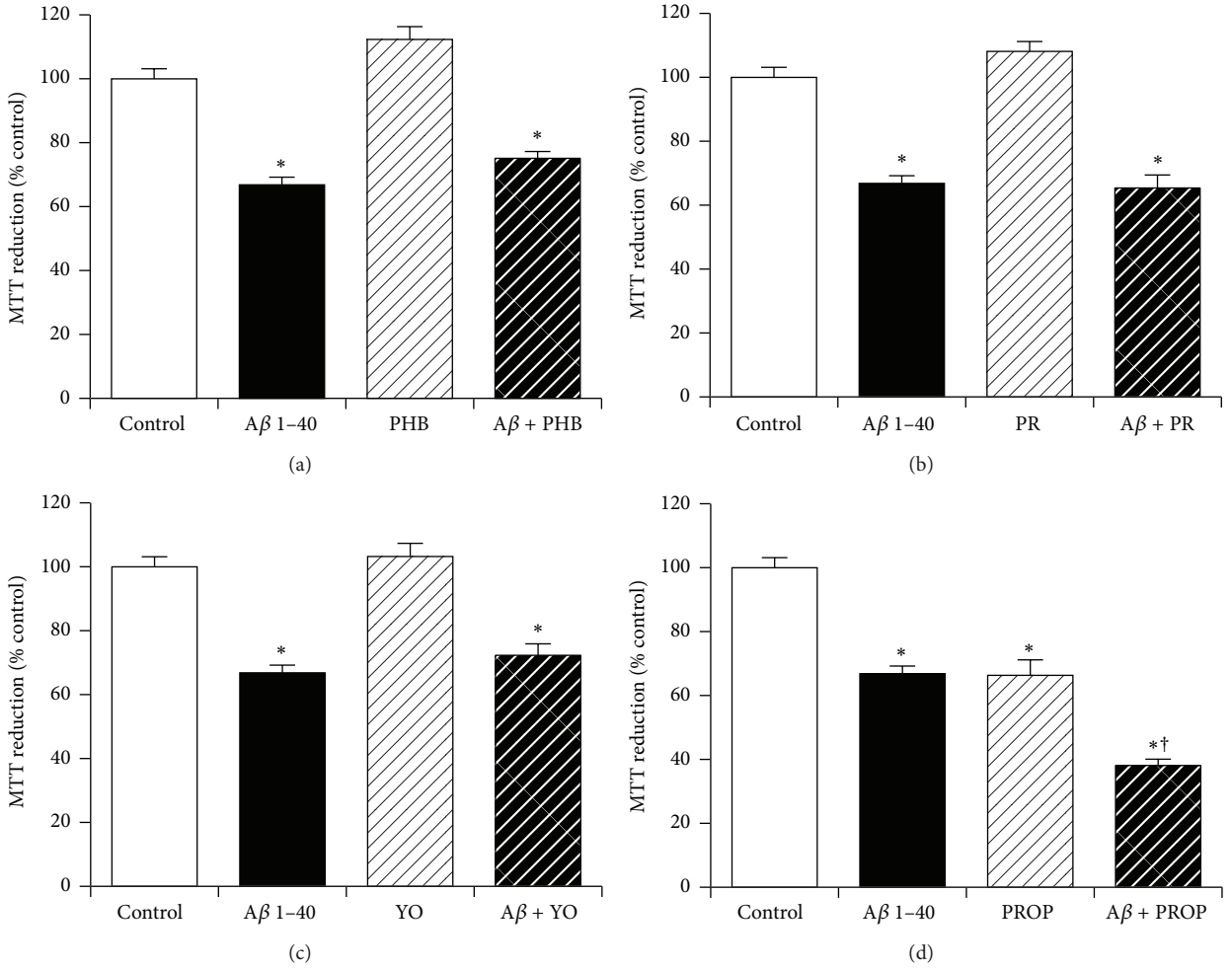

Figure 6: Effect of adrenergic receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) α-adrenergic antagonist phenoxybenzamine hydrochloride (PHB: 10 μM), (b) α-adrenergic antagonist prazosin hydrochloride (PR: 250 nM), (c) α-adrenergic antagonist yohimbine hydrochloride (YO: 50 nM), (d) β-adrenergic antagonist propranolol hydrochloride (PROP: 50 nM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

but not nicotinic cholinergic antagonists [17]. The effects of muscarinic and nicotinic cholinergic antagonists on KiSS-1 overexpression neuroprotection against Aβ were therefore tested. The doses of atropine sulphate (10 μM) and mecamylamine hydrochloride (10 μM) have previously been demonstrated to be effective in neuronal cell culture models. Results showed that the muscarinic acetylcholine antagonist atropine sulphate (Figure 7(a)) and the nicotinic acetylcholine antagonist mecamylamine hydrochloride (Figure 7(b)) had no significant effect on the toxicity of Aβ in KiSS-1 overexpressing neurons.

3.8. The Role of Dopaminergic Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP system is known to modulate dopamine levels [36] and some neurons coexpress KP plus dopamine synthesis enzymes [37]. The SH-SY5Y neuroblastoma is dopaminergic [38] and the effect of the dopaminergic antagonist haloperidol was therefore tested on KiSS-1 overexpression neuroprotection against Aβ. The dose of haloperidol (10 μM) has previously been demonstrated to be effective in neuronal cell culture models. Results showed that haloperidol had no significant effect on the toxicity of Aβ in KiSS-1 overexpressing neurons (Figure 8). The dopaminergic antagonist haloperidol has also been suggested to have neuroprotective actions against Aβ [39], an effect not observed in the KiSS-1 overexpressing neurons.

3.9. The Role of Serotonergic Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide facilitates passive avoidance learning and memory consolidation *in vivo*, which can be inhibited by 5-HT2 serotonergic antagonists [17]. The KP peptide also has antidepressant-like activity that can be inhibited by 5-HT2 serotonergic receptor antagonists [16]. The effects of serotonergic receptor antagonists on KiSS-1 overexpression neuroprotection against Aβ were therefore tested. The doses of cyproheptadine hydrochloride (10 nM) and methysergide

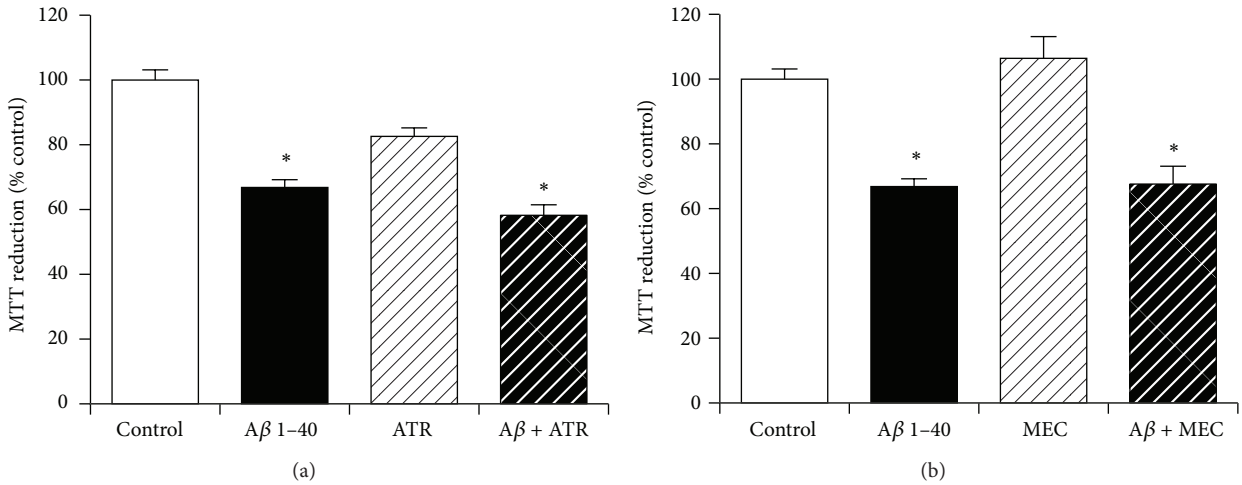

Figure 7: Effect of cholinergic receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) muscarinic acetylcholine antagonist atropine sulphate (ATR: 10 μM) or (b) nicotinic acetylcholine antagonist mecamylamine hydrochloride (MEC: 10 μM) for 2 h prior to exposure to Aβ1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^*P < 0.05$ versus control (media alone); one-way ANOVA.

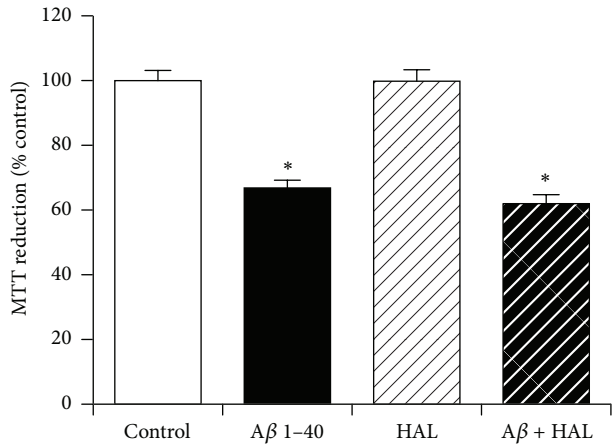

Figure 8: Effect of dopaminergic receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with haloperidol (HAL: 10 μM) for 2 h prior to exposure to Aβ1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^*P < 0.05$ versus control (media alone); one-way ANOVA.

maleate (1 μM) have previously been demonstrated to be effective in neuronal cell culture models. Results showed the 5-HT2 serotonergic antagonist cyproheptadine hydrochloride (Figure 9(a)) had no significant effect on the toxicity of Aβ in KiSS-1 overexpressing neurons. The mixed 5-HT1/5-HT2 receptor antagonist methysergide maleate caused a significant ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) reduction in the viability of KiSS-1 overexpressing SH-SY5Y neurons (Figure 9(b)), at a dose that is nontoxic to neuronal cell lines [40]. The methysergide maleate also caused a significant ($P = 0.0016$; one-way ANOVA, Dunnett post hoc test) enhancement of Aβ toxicity in the KiSS-1 overexpressing neurons (Figure 9(b)), suggesting that the toxicity of methysergide maleate in the KiSS-1 overexpressing cells was additive to the toxicity of Aβ.

3.10. The Role of GABA-A Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide facilitates passive avoidance learning and memory consolidation *in vivo*, which can be inhibited by the GABA-A antagonist bicuculline [17]. The effect of bicuculline on KiSS-1 overexpression neuroprotection against Aβ was therefore tested. The dose of 1(S),9(R)-(−)-bicuculline methiodide (50 μM) has previously been demonstrated to be effective in neuronal cell culture models. Results showed the bicuculline had no significant effect on the toxicity of Aβ in KiSS-1 overexpressing neurons (Figure 10).

3.11. The Role of Estrogen Receptor Activation in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. Activation of estrogen receptors is known to alter KP levels [41, 42] and also plays a role in the neuroprotection against Aβ [31, 43]. The effect of the estrogen receptor antagonist tamoxifen on KiSS-1 overexpression neuroprotection against Aβ was therefore tested. The dose of tamoxifen (10 μM) has previously been demonstrated to be effective in neuronal cell culture models. Results showed that tamoxifen had no significant

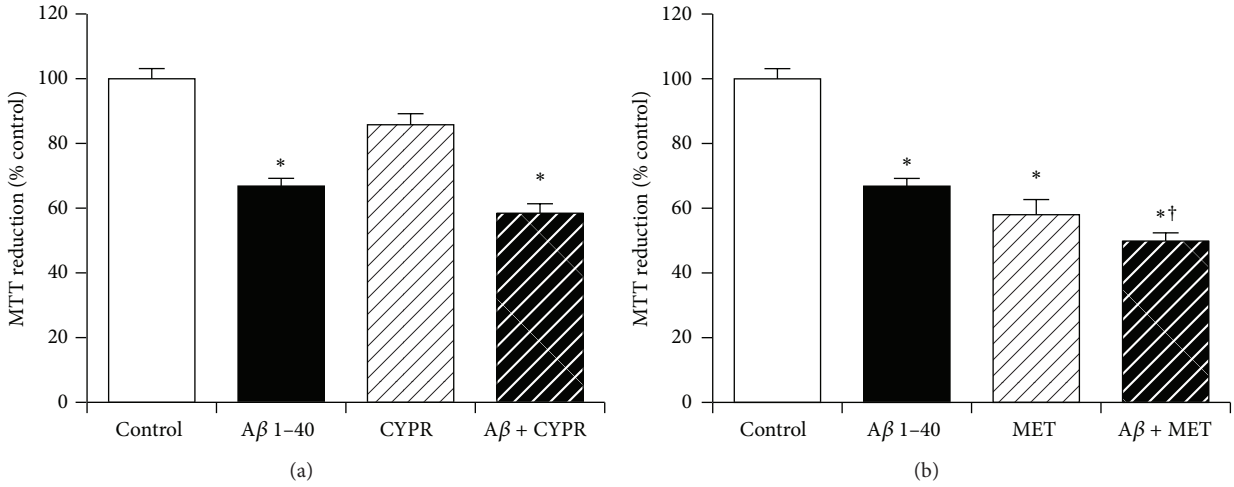

Figure 9: Effect of serotonergic receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) serotonergic antagonist cyproheptadine hydrochloride (CYPR: 10 nM) or (b) serotonergic antagonist methysergide maleate (MET: 1 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

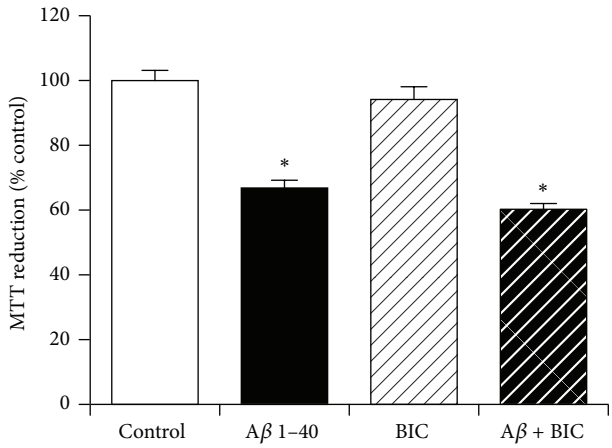

Figure 10: Effect of GABA receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with the GABA-A antagonist 1(S),9(R)-(−)-bicuculline methiodide (BIC: 50 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); one-way ANOVA.

effect on the toxicity of Aβ in KiSS-1 overexpressing neurons (Figure 11).

3.12. The Role of Catalase, Cyclooxygenase, Nitric Oxide Synthase, and γ-Secretase Enzymes in Neuroprotection against Amyloid-β by KiSS-1 Overexpression. The KP peptide is known to increase catalase activity [18], which is also neuroprotective against Aβ [44]. The KP peptide also has thermoregulatory effects [19] and acts via nitric oxide in the facilitation of passive avoidance learning plus memory consolidation *in vivo* [17]. Another possible mechanism for the neuroprotective action of KiSS-1 overexpression is via activation of intracellular second messenger pathways. The effects of catalase inhibition, cyclooxygenase inhibition, nitric oxide synthase inhibition, and also the mitogen activated protein kinase cascade inhibitor PD98059 on KiSS-1 overexpression neuroprotection against Aβ were tested to determine if these processes were involved. The doses of 3-Amino-1,2,4-triazole (50 mM), SC-560 (1 μM), N^{G}-Methyl-L-arginine acetate salt (1 mM), and PD98059 (50 μM) have previously been demonstrated to be effective in neuronal cell culture models. Results showed that catalase inhibition with 3-Amino-1,2,4-triazole had no effect on KiSS-1 overexpression neuroprotection against Aβ (Figure 12(a)). The cyclooxygenase-1 inhibitor SC-560 significantly ($P = 0.0029$; one-way ANOVA, Dunnett post hoc test) reduced KiSS-1 overexpression neuroprotection against Aβ (Figure 12(b)). Nitric oxide synthase inhibition with N^{G}-Methyl-L-arginine acetate had no effect on KiSS-1 overexpression neuroprotection against Aβ (Figure 12(c)). The mitogen activated protein kinase cascade inhibitor PD98059 caused a significant ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) reduction in the viability of KiSS-1 overexpressing SH-SY5Y neurons (Figure 12(d)), at a dose that has no effect on SH-SY5Y neurons [45]. The PD98059

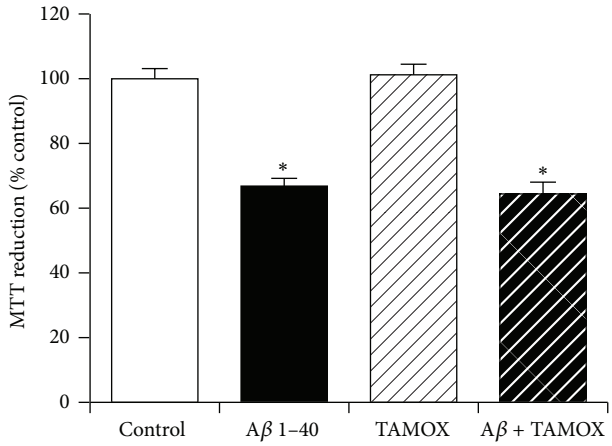

Figure 11: Effect of estrogen receptor antagonism on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with the estrogen antagonist tamoxifen (TAMOX: 10 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); one-way ANOVA.

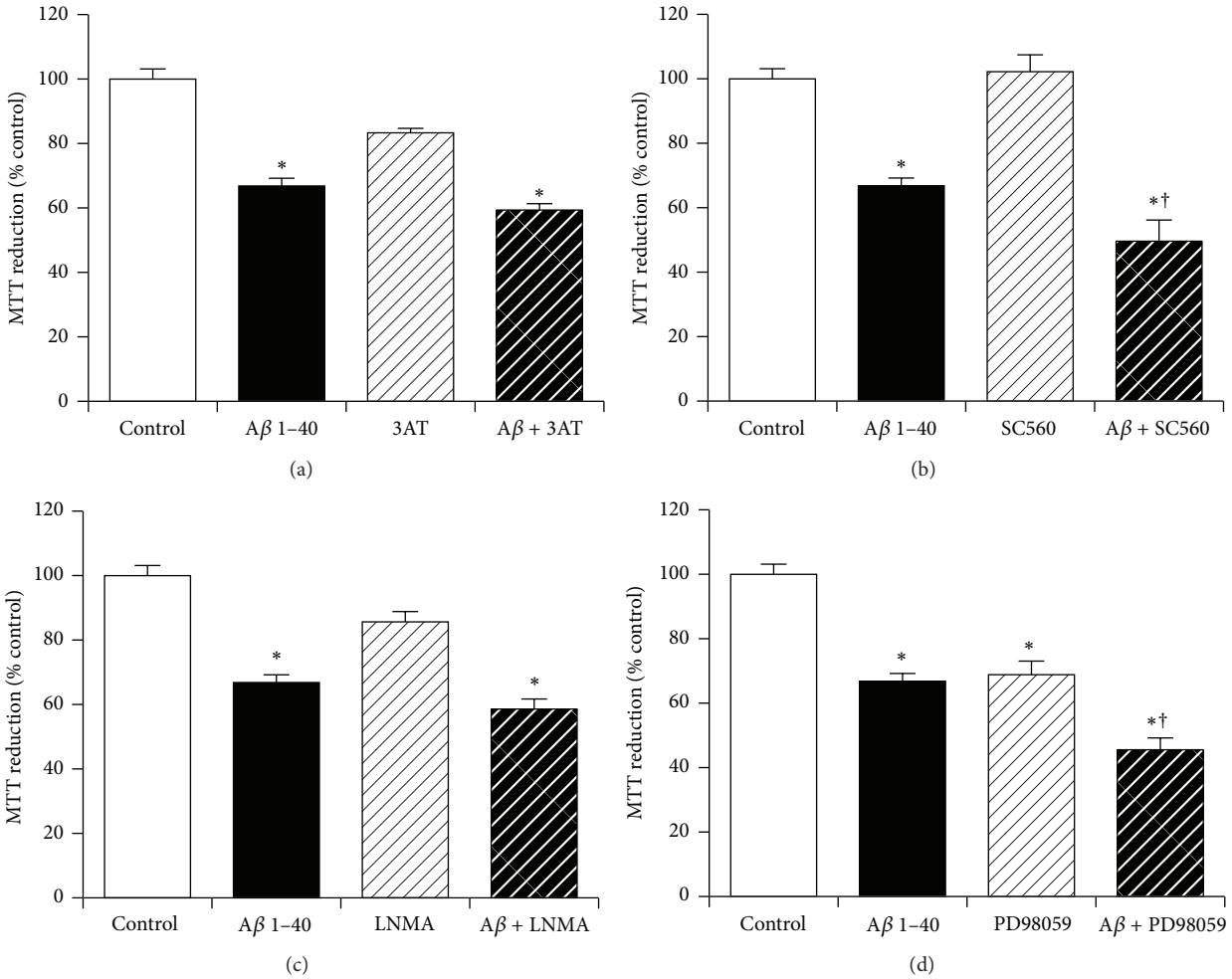

Figure 12: Effect of endogenous enzyme inhibition on KiSS-1 gene overexpression neuroprotection against amyloid-β toxicity. PKiSS cells were pretreated with (a) catalase inhibitor 3-Amino-1,2,4-triazole (3AT: 50 mM), (b) cyclooxygenase inhibitor SC560 (1 μM), (c) nitric oxide synthase inhibitor N^{G}-Methyl-L-arginine acetate salt (LNMA: 1 mM), (d) mitogen activated protein kinase cascade inhibitor PD98059 (50 μM) for 2 h prior to exposure to Aβ 1–40 (10 μM) and determination of viability by MTT reduction. Results are mean ± s.e.m. $^{*}P < 0.05$ versus control (media alone); $^{\dagger}P < 0.05$ versus Aβ alone; one-way ANOVA.

also caused a significant ($P < 0.0001$; one-way ANOVA, Dunnett post hoc test) enhancement of Aβ toxicity in the KiSS-1 overexpressing neurons (Figure 12(d)), suggesting that the toxicity of PD98059 in the KiSS-1 overexpressing cells was additive to the toxicity of Aβ rather than KiSS-1 neuroprotection being mediated via activation of the mitogen activated protein kinase cascade.

4. Discussion

The effects of the anti-KP antibody on KiSS-1 overexpression neuroprotection against Aβ have previously been reported and the mechanism of neuroprotection by KP has been suggested not to involve either the KP or NPFF receptors [1]. The failure of ASCAT and BTA-EG4 compounds, that modify KP binding to Aβ, to modulate this process suggests that the proposed binding interaction may not mediate the neuroprotection in this system. The levels of KP released by SH-SY5Y neurons in response to Aβ are likely to be insufficient to provide full neuroprotection via a binding action [1, 11]. However, in the KiSS-1 overexpressing neurons there is a significant release of an ir-KP-like material into the media that could either bind Aβ or activate receptor mediated pathways. It is therefore likely that the mechanism for neuroprotection may involve an alternative process that is more likely receptor mediated. The *in vivo* actions of KP peptides include actions on the opioid system [12, 13], oxytocin/vasopressin systems [4, 14, 15], neurotransmitter systems [16, 17], activation of endogenous antioxidants [18], activation of nitric oxide [17], and effects on thermoregulation [19] that could be mediated via the prostaglandin systems [46, 47].

The naloxone and naltrexone reduction in the toxicity of Aβ raises the possibility that endogenous opioids may play a role in the toxicity of Aβ. Similar effects were observed with naloxone and naltrexone on Aβ toxicity; however, these opioid antagonists had different effects on cell viability itself which complicated the interpretation of the results. The antiopioid activity of KP peptides has been suggested by their activation of NPFF receptors [8, 9] and the KiSS-1 derivative KSO also acts as an NPFF ligand [10]. However, the NPFF antagonist RF9 had no effect on KiSS-1 overexpression neuroprotection against Aβ. The RF9 is known to block the antiopioid activity of NPFF [48] but has recently been suggested to be ineffective at blocking all the actions of NPFF and related peptides [49]. As such the effects of KiSS-1 overexpression on Aβ toxicity are unlikely to involve a partial suppression of endogenous opioid actions by KP that is enhanced by naloxone or naltrexone.

The effects of atosiban suggest a role for the oxytocin system in the neuroprotection provided by KiSS-1 overexpression. The actions of atosiban also include inhibition of vasopressin receptors [50] and it is known that some of the actions of KP peptides are mediated via actions on vasopressin [14]. *In vivo* KP activates both oxytocin and vasopressin [4, 14, 15], as such it is possible that either or both the oxytocin and vasopressin systems are involved in KiSS-1 neuroprotection.

From this study the adrenergic, cholinergic, dopaminergic, serotonergic, and GABA neurotransmitter systems plus the nitric oxide and estrogen receptor activated systems do not appear to play a role in the neuroprotective actions of KiSS-1 overexpression against the Aβ peptide. The β-adrenergic antagonist propranolol hydrochloride and the mixed 5-HT1/5-HT2 receptor antagonist methysergide maleate both had toxic actions in KiSS-1 overexpressing neurons at concentrations that are not toxic to SH-SY5Y neurons [35, 40]. The β-adrenergic antagonist propranolol hydrochloride and the mixed 5-HT1/5-HT2 receptor antagonist methysergide maleate also enhanced Aβ toxicity; however, this is more likely due to the toxicity of these antagonists to KiSS-1 overexpressing neurons rather than the involvement of noradrenaline or serotonin in the KiSS-1 mediated neuroprotection. Both noradrenaline [51, 52] and serotonin [53] have neuroprotective properties. The mitogen activated protein kinase cascade inhibitor PD98059 also inhibited cell viability and the β-adrenergic [54] plus 5HT1 serotonergic [55] receptors can act via the mitogen activated protein kinase cascade. Since the KP peptide is known to activate both β-adrenergic and serotonergic pathways *in vivo* [17] it is possible that these pathways are upregulated in this overexpression system and play a role in the neuronal survival. The mitogen activated protein kinase cascade may provide the second messenger system for the β-adrenergic plus 5HT1 serotonergic pathways involved.

The mitogen activated protein kinase cascade inhibitor PD98059 has previously been shown to reduce the anti-Aβ effects of a number of neuroprotective compounds [56–60]. PD98059 also attenuates KP induced modulation of GnRH mRNA [61] and KP upregulation of excitatory synaptic transmission [62].

The SC-560 cyclooxygenase-1 inhibitor has previously been shown to reduce Aβ production in an AD model [63]. The specificity of SC-560 for cyclooxygenase-1 over cyclooxygenase-2 is altered in some cell systems [64] and it is unknown which form of cyclooxygenase contributes in the KiSS-1 overexpression model. The ability of this compound to enhance Aβ toxicity in the KiSS-1 overexpression model suggests that there may be modulation of the cyclooxygenase system in these neurons. The ability of KP to modulate thermoregulatory responses *in vivo* [19] could be modulated via cyclooxygenase inhibitors [46, 47]. As such KP could be acting via prostaglandin synthesis in this overexpression model and *in vivo*.

The observation that KiSS-1 neuroprotection has both an oxytocin/vasopressin plus a cyclooxygenase dependent component could be due to endogenous oxytocin or vasopressin activating cyclooxygenase. Both *in vivo* adminstration and *in vitro* administration of oxytocin [65, 66] or vasopressin [67, 68] causes an activation of prostaglandin synthesis that is cyclooxygenase dependent. The SH-SY5Y neuronal cell line is known to express the vasopressin gene [69] suggesting that this could be a source of the endogenous material antagonized by atosiban. This proposed mechanism for KiSS-1 mediated neuroprotection against Aβ is summarized in Figure 13. The action of KP or KSO products of the KiSS-1 gene appears to be independent of the KP and NPFF receptors and a direct binding action on Aβ cannot be excluded [1] but at the concentrations of KP found in the system is unlikely to have a major effect.

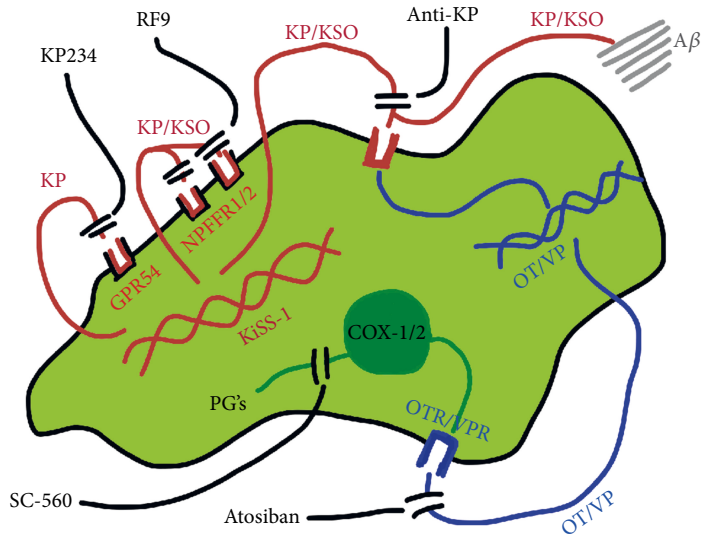

Figure 13: Model for KiSS-1 gene overexpression neuroprotective mechanisms against amyloid-β toxicity. KP: kisspeptin; KSO: kissorphin; KP234: kisspeptin receptor antagonist; GPR54: kisspeptin receptor; RF9: neuropeptide FF receptor antagonist; NPFFR1/2: neuropeptide FF receptors 1 and 2; Anti-KP: anti-kisspeptin 45–54 antiserum; Aβ: amyloid-β; OT: oxytocin; VP: vasopressin; atosiban: oxytocin/vasopressin receptor antagonist; OTR: oxytocin receptor; VPR: vasopressin receptor; COX-1/2: cyclooxygenase 1 and 2; PG's: prostaglandins; and SC-560: cyclooxygenase 1 antagonist.

5. Conclusion

KiSS-1 overexpression is neuroprotective against Aβ and the action appears to involve the KP peptide product of KiSS-1 processing, which is released by the cells. The mechanism of neuroprotection does not involve the KP or NPFF receptors. Opioids play a role in the toxicity of Aβ in the KiSS-1 overexpression system. The mechanism of protection appears to have an oxytocin/vasopressin plus a cyclooxygenase dependent component, which may be linked and can be blocked by the oxytocin/vasopressin antagonist atosiban or the cyclooxygenase-1 antagonist SC-560 (Figure 13). The contribution of KP binding to Aβ may also contribute to the neuroprotection observed in this model [1].

Conflict of Interests

N. G. N. Milton is named as the inventor on patent applications filed by the University of Roehampton for the use of kissorphin peptides to treat Alzheimer's disease, Creutzfeldt-Jakob disease, or diabetes mellitus (Publication Nos. GB2493313 A, WO 2011/144714 A1, and EP 2 388 012 A1); under the University of Roehampton rules he could benefit financially if these patents are granted and commercially exploited.

Authors' Contribution

N. G. N. Milton and A. Chilumuri conceived and designed the experiments, performed the experiments, and analyzed the data. N. G. N. Milton wrote the paper. N. G. N. Milton and A. Chilumuri critically reviewed the paper.

Acknowledgments

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